Cell type–specific regulation of von Willebrand factor expression by the E4BP4 transcriptional repressor

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Mechanisms of tissue-restricted patterns of von Willebrand factor (VWF) expression involve activators and repressors that limit expression to endothelial cells and megakaryocytes. The relative transcriptional activity of the proximal VWF promoter was assessed in VWF-producing and -nonproducing cells, and promoter activity was highest in endothelial cells followed by megakaryocytes. Only basal VWF promoter activity was seen in nonendothelial cells. Here we identify a negative response element located at nucleotides (nts) +96/+105 and demonstrate, using chromatin immunoprecipitation (ChIP) analysis, that in vivo this sequence interacts with the E4BP4 transcriptional repressor. Differences in size and relative abundance of nuclear E4BP4 were observed. In HepG2 cells, low levels of larger forms of E4BP4 are present that directly interact with the negative response element. In VWF-expressing cells, high levels of smaller forms predominate with no evidence of direct DNA binding. However, in endothelial cells, mutation of the VWF E4BP4 binding motif not only restores but also further elevates VWF promoter activity, suggesting that E4BP4 may be part of a coordinated binding complex. These observations implicate this binding motif in repressing both activated and basal levels of VWF transcription by different cell type–specific mechanisms, and support the hypothesis that E4BP4 sequesters negative regulators of transcription, thereby enhancing activated gene expression. (Blood. 2005;105:1531-1539)

Introduction

Von Willebrand factor (VWF) is synthesized exclusively in endothelial cells and megakaryocytes. It mediates the interaction between platelets and components of the subendothelium at sites of vascular injury, and in the circulation protects the coagulation factor VIII from proteolytic degradation. The critical role that VWF plays in normal hemostasis is illustrated by the effects of abnormally low or high plasma VWF levels. Deficiencies of VWF result in the bleeding diathesis von Willebrand disease, while elevated levels are associated with pathophysiologic processes such as coronary artery thrombosis. There is considerable variation in plasma VWF levels between individuals, but as well, within an individual there is a marked regional heterogeneity in VWF expression in endothelial cells throughout the vasculature. Significant efforts have been directed at understanding transcriptional regulatory mechanisms that control not only cell lineage–specific expression but also the observed variations in VWF levels.

Initial characterization of the 5′-flanking region of the VWF promoter identified the presence of positive and negative regulatory elements as far upstream as 2 kilobases (kb) from a single transcriptional start site. The structural organization and nucleotide sequence of the human, bovine, and murine proximal VWF promoters, including the first exon, are quite similar. In vitro analysis of the human VWF promoter identified a minimal core promoter between nucleotides (nts) −90 and +22 that is capable of inducing transcription in both endothelial and nonendothelial cells. In vitro, the region that spans nts −487 to +247 imparts endothelial-specific promoter activity but is repressed in all cell types by an upstream element located at nts −487/−440 that interacts with an NF-1–like transcriptional repressor. Enhanced transcription of the VWF gene is controlled in part by the positive regulatory region (+145 to +247) of the promoter that also contains GATA consensus binding sites at nt +220 and nt −80. These elements relieve the transcriptional inhibition of the upstream negative regulatory element in an endothelial cell–specific manner. A putative GATA binding element has also been identified at nt +53 of the VWF promoter. Members of the Ets family of transcription factors bind to the consensus site located at nt −56 and also up-regulate VWF promoter activity in both endothelial cells and HeLa cells. An additional negative regulatory element has been localized between nts −133 and −125 that binds the Oct-1 transcription factor. Finally, the NFY transcription factor serves either as an activator or a repressor of VWF promoter activity. NFY acts as an activator when it binds to its consensus sequence at nt −18 of the promoter, but functions as a repressor in nonendothelial cells through the recruitment of histone deacetylase to sequences +226 to +234. This region of the promoter also interacts with GATA6, which trans-activates the VWF promoter in an endothelial-specific manner.

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Transgenic mouse models have been used to elucidate the underlying mechanism that confers endothelial-specific expression of VWF in vivo. Surprisingly, the region spanning nt −487 and +246 that limits expression to endothelial cells in vitro, directs expression only in blood vessels of the brain. Even the inclusion of an additional 2 kb of 5′ flanking sequences confers expression only to the endothelium of the brain, heart, and skeletal muscle, suggesting that additional regions of the VWF locus are required to direct ubiquitous endothelial expression of VWF.

Clearly, the molecular mechanisms involved in regulating VWF expression in endothelial cells are complex. It has been proposed that VWF is regulated in a modular fashion in so far as its expression is mediated by the overall interaction of distinct signaling pathways that interact with different regions of the VWF promoter. With a view to better understand the cell lineage-specific nature of VWF expression, we studied the proximal promoter region of the VWF gene in bovine aortic endothelial cells (BAECs), differentiated and undifferentiated megakaryocytic cells (Dami), and a non-VWF-expressing cell line, HepG2. Here we describe the presence of a transcriptional repressor element located between ns +96 and +105 and show that this cis-acting element interacts with the adenovirus E4 promoter-binding protein 4 (E4BP4) transcription repressor to regulate VWF promoter activity.

Materials and methods

Cell culture

The human megakaryoblastic cell line Dami, hepatoma cell line HepG2, baby hamster kidney cell line BHK, and the human epitheloid carcinoma cell line, HeLa, were all obtained from the American Type Culture Collection (ATCC, Rockville, MD). Human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics (Walkersville, MD). HepG2, HUVEC, and HeLa cell lines were grown in minimal essential media (MEM); BHK cells were grown in Dulbecco modified Eagle medium (DMEM); and all cultures were supplemented with 10% heat-inactivated fetal bovine serum. Both undifferentiated Dami and differentiated Dami cells were maintained in Iscoves modified Dulbecco medium (IMDM) supplemented with 10% heat-inactivated horse serum. To induce undifferentiated Dami cells to differentiate, 5% DMSO or 50 nM phorbol myristate acetate (PMA) with 0.25% DMSO.27

Generation of the mutated E4BP4 binding site

The TAA (nts +102 to +104) of the E4BP4 binding site in the VWF promoter was converted to CTG by site-directed mutagenesis. A 340-bp region of the VWF promoter between the XhoI and HindIII was subcloned into the pBK-CMV vector (Stratagene, La Jolla, CA). Site-directed mutagenesis was performed using the Quikchange Multi-Site Directed Mutagenesis Kit (Stratagene) according to the manufacturer’s protocol.

Cell transfection and luciferase assay

Large-scale DNA preparations of VWF reporter constructs were purified using Qiagen columns (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Dami cells were transfected using Effectene Reagent (Qiagen) with 550 ng of the VWF reporter construct and 50 ng of an expression vector for β-galactosidase (pCMV-βGAL). Transfections of BAECs and BHK, HeLa, and HepG2 cells were carried out by the calcium phosphate coprecipitation method29 in 35-mm2 plates using 2.5 μg of the VWF reporter constructs and 0.5 μg of pCMV-βGAL. Luciferase activity (Promega Luciferase Assay) and β-galactosidase activity (Tropic Galacto-Light System; Applied Biosystems, Bedford, MA) were measured according to the manufacturer’s instructions using a Perkin Elmer Luminescence Reader (Stratagene, La Jolla, CA). Luciferase activity was normalized with respect to β-galactosidase activity to correct for differences in transfection efficiencies and cell numbers. The E4BP4 expression construct was a kind gift from Dr Helen Hurst (Hammersmith Hospital, London, United Kingdom).

Gel mobility shift assays

Double-stranded oligonucleotides were designed for the E4BP4 consensus-binding site, the VWF E4BP4 binding region (Neg 5), and the mutated VWF E4BP4 binding region (Neg 5 Mut) (Table 3) and labeled with 15 μCi (0.555 MBq) [32P] deoxyadenosine triphosphate (dATP). Nuclear protein extracts from BAECs, HUVECs, and HepG2 cells were prepared using the method of Dignam30 and from Dami cells by the method of Sierra.31 Extracts (5-10 μg) were incubated at room temperature for 20 minutes in binding buffer (25 mM HEPES [N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid], pH 7.6; 5 mM MgCl2, 34 mM KCl, 0.05 μg/mL poly poly[deoxyinosinic-deoxyxycytidylic] acid sodium salt [dIdC], 1 μg/mL bovine serum albumin) and 100 000 cpm 32P-labeled probe. In competition studies, a 100-fold molar excess of unlabeled double-stranded oligonucleotide was also included in the binding reaction. For the antibody supershift assays, nuclear protein was incubated at room temperature for 15 minutes with 4 μg of an antihuman E4BP4 antibody (no. SC0549X; Santa Cruz Biotechnology, Santa Cruz, CA) after the initial incubation with the labeled oligonucleotide probe. Preimmune serum was used as a control for nonspecific binding of the antibody.

Table 1. Primer sets used to create VWF promoter deletion constructs

<table>
<thead>
<tr>
<th>PGL2 basic constructs (S/AS)</th>
<th>Primer sequence (5′-3′)</th>
</tr>
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<tbody>
<tr>
<td>VWF-Prom (−91-S)</td>
<td>AAACTTTTTATACCTTTGAG</td>
</tr>
<tr>
<td>VWF-Prom (−229-AS)</td>
<td>GCGCTATTCCTCGAGGCTCTT</td>
</tr>
<tr>
<td>VWF-Prom (−63-S)</td>
<td>AAATCCATTCTTCTACATTG</td>
</tr>
<tr>
<td>VWF-Prom (−247-AS)</td>
<td>CCCCTGCAAAATGCTGGC</td>
</tr>
<tr>
<td>VWF-Prom (−63-S)</td>
<td>AAATCCATTCTTCTACATTG</td>
</tr>
<tr>
<td>VWF-Prom (−169-AS)</td>
<td>GGTCATCTTCCAGTCTGC</td>
</tr>
<tr>
<td>VWF-Prom (−63-S)</td>
<td>AAATCCATTCTTCTACATTG</td>
</tr>
<tr>
<td>VWF-Prom (−70-AS)</td>
<td>GGCGGATAAGAAGCCAAAG</td>
</tr>
<tr>
<td>VWF-Prom (−53-S)</td>
<td>TTGGCTATTTATCTCCCCAC</td>
</tr>
<tr>
<td>VWF-Prom (−229-AS)</td>
<td>GCGCTATTCCTCGAGGCTCTT</td>
</tr>
<tr>
<td>VWF-Prom (−53-S)</td>
<td>TTGGCTATTTATCTCCCCAC</td>
</tr>
<tr>
<td>VWF-Prom (−169-AS)</td>
<td>GGTCATCTTCCAGTCTGC</td>
</tr>
<tr>
<td>VWF-Prom (−53-S)</td>
<td>TTGGCTATTTATCTCCCCAC</td>
</tr>
<tr>
<td>VWF-Prom (−145-AS)</td>
<td>GGCCTACAGCTGTCATC</td>
</tr>
<tr>
<td>VWF-Prom (−170-S)</td>
<td>AGACAGGCTGCAATGTTG</td>
</tr>
<tr>
<td>VWF-Prom (−229-AS)</td>
<td>GCGCTATTCCTCGAGGCTCTT</td>
</tr>
<tr>
<td>VWF-Prom (−63-S)</td>
<td>TTGGCTATTTATCTCCCCAC</td>
</tr>
<tr>
<td>VWF-Prom (−102-AS)</td>
<td>TGTACGGAGCTGCTOZ2A</td>
</tr>
<tr>
<td>VWF-Prom (−83-S)</td>
<td>TCCACACCCCGTCGCTAC</td>
</tr>
<tr>
<td>VWF-Prom (−121-AS)</td>
<td>CGGCACTCTTCTCTCTTTAG</td>
</tr>
</tbody>
</table>

S indicates sense; AS, antisense.
Table 2. Complementary oligonucleotides used to create single-site reporter constructs

<table>
<thead>
<tr>
<th>PGL2 promoter constructs</th>
<th>Annealed oligonucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>VWF E4BP4 (Neg 5)</td>
<td>5’ GATC GGCCTATCACACCCCCAGTTAATTG GATC 5’</td>
</tr>
<tr>
<td>Mutant VWF E4BP4 (Neg 5 Mut)</td>
<td>5’ GATC GGCCTATCACACCGCA CCGAGTGTACACCTG GATC 5’</td>
</tr>
<tr>
<td>E4BP4 consensus</td>
<td>5’ GATC GTTATGTAACG CATACATTGCC GATC 5’</td>
</tr>
</tbody>
</table>

Western blot analysis

Nuclear protein samples were diluted into loading buffer that contained reducing agent and separated on a sodium dodecyl sulfate (SDS)–polyacrylamide gel (7.5%) in Tris (tris(hydroxymethyl)aminomethane)–glycine buffer. Molecular mass markers were purchased from New England Biological (catalog no. 7707S; Beverly, MA) and Bio-Rad (catalog no. 161-0374; Mississauga, ON). After electrophoresis, immunoblotting was performed with a polyclonal anti-E4BP4 antibody (nos. SC9550; Santa Cruz Biotechnology). Preincubating the antibody with its blocking peptide prior to immunoblotting was carried out to assess the specificity of the E4BP4 antibody. Blots were incubated with affinity-purified anti–rabbit immunoglobulin G (IgG) horseradish peroxidase–conjugated secondary antibody (Affinity Biological, Hamilton, ON).

Chromatin immunoprecipitation (ChiP) assay

The ChiP assay was carried out using the ChiP Assay Kit from Upstate Biotechnology (Lake Placid, NY) as recommended by the manufacturer. Total chromatin was removed prior to immunoprecipitation to serve as an input sample. Immunoprecipitation using anti-E4BP4 antibodies (nos. SC9550 and SC9549X; Santa Cruz Biotechnology) was performed at 4°C for 16 hours. A negative control without antibody was also carried out. Human VWF and β-actin promoter sequences were amplified using primer pairs and conditions outlined in Table 4.

Amplification of the E4BP4 RNA

Total RNA was isolated from the various cell types using TRIzol Reagent (Life Technologies, Bethesda, MD) according to the manufacturer’s instructions. Reverse transcription and PCR amplification were carried out using the primers and conditions outlined in Table 4.

Results

Relative transcriptional activity of the VWF proximal promoter in VWF-expressing and non–VWF-expressing cells

To characterize the VWF promoter region that encompasses the 2 GATA elements at nts −80 and +220, and to identify elements that influence transcription, various deletion constructs spanning this region were engineered (Figure 1A). To assess the cell type–specific relative transcriptional activity of the proximal promoter, the region spanning nts −91 to +229 was transfected into VWF-expressing and -nonexpressing cells. (Figure 1B). This region of the promoter is in the order of 40-fold more transcriptionally active in endothelial cells (BAECs) compared with nonendothelial cells (HepG2, BHK, and HeLa), and in undifferentiated megakaryocytes it is about 4-fold less than that seen in the endothelial cells. However, once Dami cells are induced to differentiate, the promoter activity more than doubles.

Identification of repressor activity in the first noncoding exon of the VWF gene

The VWF promoter activity of the various deletion constructs, relative to the construct containing nts −91 to +229, was assessed in endothelial cells, undifferentiated and differentiated megakaryocytes, and non–VWF-expressing HepG2 cells (Figure 1C). Deletion of the GATA element located at nt −80 resulted in a loss of transcriptional activity only in VWF-producing cells. In HepG2 cells, deletion of this element resulted in an increase in transcriptional activity. Deletion of most of the positive regulatory region (+169 to +247) resulted in a significant loss of promoter activity in all cell types analyzed. Additional deletion of DNA sequences between nts +70 and +169 resulted in a significant increase in promoter activity only in the cells that synthesize VWF, suggesting the presence of a cell type–specific repressive element.

Table 3. Sequence information of the oligonucleotides used in electrophoretic mobility shift assays

<table>
<thead>
<tr>
<th>Site</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E4-BP4 consensus</td>
<td>GGTACGTCATGCATGCATGC ACCGATCC</td>
</tr>
<tr>
<td>NEG 5</td>
<td>GGTACGTCATGCATGCATGC ACCGATCC</td>
</tr>
<tr>
<td>NEG 5 Mut</td>
<td>GGTACGTCATGCATGCATGC ACCGATCC</td>
</tr>
</tbody>
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considered statistically significant when compared with a control construct were assessed using a Student t test. The mean fold increase in normalized luciferase expression of the promoter construct containing nts 91 to 229, relative to the promoterless pGL2 Basic construct, transfected into VWF-/H11001 producing cells. The error bars indicate the standard error of the mean. Statistical differences between each deletion construct and the pGL2 control construct are indicated on each graph. The error bars indicate the standard error of the mean. Statistical differences between each deletion construct and the pGL2 control construct are indicated on each graph. The error bars indicate the standard error of the mean. Statistical differences between each deletion construct and the pGL2 control construct are indicated on each graph. The error bars indicate the standard error of the mean. Statistical differences between each deletion construct and the pGL2 control construct are indicated on each graph. The error bars indicate the standard error of the mean. Statistical differences between each deletion construct and the pGL2 control construct are indicated on each graph. The error bars indicate the standard error of the mean. Statistical differences between each deletion construct and the pGL2 control construct are indicated on each graph. The error bars indicate the standard error of the mean. Statistical differences between each deletion construct and the pGL2 control construct are indicated on each graph. The error bars indicate the standard error of the mean. Statistical differences between each deletion construct and the pGL2 control construct are indicated on each graph. The error bars indicate the standard error of the mean. Statistical differences between each deletion construct and the pGL2 control construct are indicated on each graph. The error bars indicate the standard error of the mean. Statistical differences between each deletion construct and the pGL2 control construct are indicated on each graph. The error bars indicate the standard error of the mean. Statistical differences between each deletion construct and the pGL2 control construct are indicated on each graph. The error bars indicate the standard error of the mean. Statistical differences between each deletion construct and the pGL2 control construct are indicated on each graph. The error bars indicate the standard error of the mean. Statistical differences between each deletion construct and the pGL2 control construct are indicated on each graph. The error bars indicate the standard error of the mean. Statistical differences between each deletion construct and the pGL2 control construct are indicated on each graph. The error bars indicate the standard error of the mean. Statistical differences between each deletion construct and the pGL2 control construct are indicated on each graph.

Localization of a putative repressor element to nucleotides +96/+105

To further delineate the location of the repressor activity, 2 reporter constructs were generated that encompass nts +63 to +121, cloned upstream of the SV40 promoter. These constructs were compared for transcriptional activation in VWF-producing and -nonproducing cells. The construct containing nts +83 to +121 of the VWF promoter exhibited a 3.5- and 2.3-fold reduction in SV40 promoter activity in BAEC and Dami cells, respectively, while only a 1.5-fold reduction in activity was observed in HepG2 cells. The construct containing +63 to +102 showed increased repression when compared with +83 to +102 in HepG2 cells, however the difference was not statistically significant.

The nucleotide sequence spanning +83 to +121 was subjected to a transcription factor binding site homology search using the database located at http://www.cbrc.jp/research/db/TFSEARCH.html, and a putative element between nts +96/+105 (in the reverse orientation) was identified that bore close homology to an E4BP4 consensus binding site (Figure 3B) with a one-base pair mismatch of the 10-base pair consensus sequence. E4BP4 was first characterized by Cowell and Hurst and Cowell as a ubiquitously expressed transcriptional repressor, and a novel member of the basic/leucine zipper (b-ZIP) family of transcription factors.

The E4BP4 transcriptional repressor binds to the cis-element at nucleotides +96/+105 in HepG2 cells and represses basal VWF promoter activity

To investigate the protein-binding properties of the putative repressor element, a series of double-stranded oligonucleotides were generated (Table 3). The Neg 5 oligonucleotide contains the putative VWF E4BP4 element, while Neg 5 Mut contains the mutated putative VWF E4BP4 element (nts +102 to +104 mutated from TAA to CTG). Electrophoretic mobility shift assays (EMSA) and supershift assays were performed with nuclear proteins derived from HepG2 cells transfected with an E4BP4 expression plasmid, and untransfected HepG2 cells, and the results are presented in Figure 4A and 4B, respectively. The transfected HepG2 cells were used as a positive control for E4BP4 nuclear protein. Labeled oligonucleotides corresponding to the E4BP4 binding site consensus and the putative VWF E4BP4 site show a similar smear of gel-retarded complexes. This smear is expected as bZIP transcription factors undergo extensive homo- and heterodimerization, and recognize similar DNA binding elements. Neg 5 Mut does not show a similar smear of gel-retarded DNA/protein complexes (Figure 4C). The supershift assays show slower migrating complexes with both the Neg 5 and E4BP4 oligonucleotides, which clearly indicate that the E4BP4 directly interacts with the VWF E4BP4 binding element. The presence of a very faint complex in the non–E4BP4-transfected HepG2 cells mirrors the basal level of the endogenous VWF promoter activity in these cells and shows that E4BP4 is a minor component of the bZIP proteins that interact with these DNA sequences. Similar results have been reported for the E4BP4 consensus sequence. Replacement of the E4BP4 antibody with preimmune serum confirms that the supershifted
DNA/protein complex is not a nonspecific complex (Figure 4D). Competition binding assays using a 100-fold molar excess of unlabeled E4BP4, Neg 5, and Neg 5 Mut as a competitor for binding were carried out with nuclear proteins isolated from HepG2 cells (Figure 4E). When Neg 5 was used as the labeled probe, both E4BP4 and Neg 5, but not Neg 5 Mut, were able to efficiently compete away the gel-retarded DNA/protein complexes. Similar results were seen when the E4BP4 consensus sequence was used as the labeled probe.

To confirm that the VWF E4BP4 element is able to function as a cis-acting element through which the E4BP4 transcriptional repressor can mediate inhibition of transcription, several reporter constructs were generated for transfection into HepG2 cells. Constructs contain one copy of either the VWF E4BP4 element, the E4BP4 consensus element, nts +63/+121 of the VWF promoter, or nts +83/+121 of the VWF promoter, cloned upstream of the luciferase gene, regulated by the SV40 promoter. Similar results were seen when the E4BP4 consensus sequence was used as the labeled probe.

Analysis of DNA binding of E4BP4 to the VWF promoter negative response element in VWF-producing cells

The E4BP4 binding element in the VWF promoter mediates the strongest repressive effect in endothelial cells. In BAECs, removal of the element restores transcription (5-fold) to levels seen only with the full-length proximal VWF promoter, which, unlike the deleted construct, contains additional GATA elements at −81 and +220 (Figure 1C). In differentiated and undifferentiated megakaryocytes, removal of the negative element results in 3- and 2-fold increases, respectively, in promoter activity. It would appear, then, that the negative response element mediates a restraint on transcription in VWF-producing cells. To investigate the protein-binding properties of E4BP4 to the negative response element, standard EMSA analysis using nuclear proteins isolated from VWF-expressing cells (Dami and BAECs) and the oligonucleotides containing the consensus E4BP4 and VWF E4BP4 binding sites was carried out. The results presented in Figure 5A show a diffuse smear of protein/DNA complexes, but this was evident only when 10 μg nuclear protein was used with a prolonged exposure of the autoradiograph. No supershifted complex could be detected when the E4BP4 antibody was used. Nuclear proteins isolated from HUVECs also showed only negligible DNA binding with no supershifted complex (data not shown).

Western blot analysis of nuclear proteins

To determine if E4BP4 is present in the nucleus of VWF-producing cells, a Western blot was prepared with nuclear proteins isolated from HepG2 cells, BAECs, HUVECs, and Dami cells, and the results are shown in Figure 5B. Preincubating the antibody with its blocking peptide assessed nonspecific binding of the E4BP4 antibody to the nuclear proteins. Nonspecific bands of 35 and 62 kDa are observed. In HepG2 nuclear extracts, however, an intense band corresponding to a molecular mass of about 53 kDa is observed,
which was the reported mass when E4BP4 was first identified. In nuclear extracts isolated from undifferentiated and differentiated Dami cells, an intense band of approximately 33 kDa is observed, and this size of E4BP4 has never previously been reported. No significant difference in the amount of E4BP4 is observed between the 2 cell types, indicating that it may not play a prominent role in modulating the levels of VWF expression when Dami cells are induced to differentiate. Apparently, only a very small amount of higher molecular weight E4BP4 is present in the nucleus of HUVECs. To determine if these forms of E4BP4 result from alternative splicing, and to evaluate the presence of the DNA binding domain, RNA was isolated from the various cell types and reverse transcribed and a fragment spanning nts 2 to 1521 was amplified (Figure 5C). The nucleotide sequence of the bovine E4BP4 5’ untranscribed region (UTR) is not known and presumably diverges from the human sequence as all attempts to amplify the E4BP4 RNA from BAECs failed. A second fragment that spans nts 562 to 1521 of the only translated exon was therefore amplified from BAECs and compared with the identical fragment amplified from HUVECs. The 1.51-kb fragment isolated from HUVECs was sequenced and the predicted DNA binding motif was identified. The other amplified fragments were all assessed by restriction digest analysis. No evidence of alternative splicing was detected, suggesting that posttranslational modifications of E4BP4 (including possible proteolysis) must occur to account for the different E4BP4 forms seen in these studies.

Mutation of the VWF E4BP4 binding element restores VWF promoter activity

To determine if the VWF E4BP4 binding element is involved in the repression of transcription in the VWF-producing cells, the VWF E4BP4 binding motif was mutated in the construct that spans nts −63 to +169, and the effect on promoter activity was assessed in BAECs (Figure 6A). A 5-fold increase in promoter activity was observed with the mutated VWF E4BP4 element relative to the wild-type element. Furthermore, the relative promoter activity was 2.6-fold higher than that observed with the full-length −91 to +229 construct, which contains both trans-activating GATA elements at nts −80 and +220. This clearly indicates that the VWF E4BP4 element is involved in repression of activated VWF promoter activity. To confirm the involvement of E4BP4 and the negative response element in repressing basal levels of VWF promoter activity, the effect of mutating the E4BP4 binding element was assessed in HepG2 cells. Only a modest relief of repression was observed (Figure 6C). However, since relatively low levels of nuclear E4BP4 are present in HepG2 cells (Figure 5B) and since the extent of repression appears to be related to the amount of nuclear E4BP4 (Figure 4F), the mutant construct was compared with the identical construct that contained the E4BP4 element. A t test assessed for significant repression between cotransfection of the reporter construct in the absence of the E4BP4 expression vector. Error bars represent standard error of the mean. A t test assessed for significant repression between cotransfection of the reporter construct in the absence or presence of the E4BP4 expression vector. Error bars represent standard error of the mean. A significant increase (P < .0001) in VWF promoter activity relative to HepG2 cells that were not cotransfected with the recombinant E4BP4 was observed. Furthermore, a significant increase (P = .0007) in transcriptional activity was observed when the mutated E4BP4 binding element was compared with the identical construct that contained the wild-type E4BP4 element (Figure 6E).

E4BP4 interacts with the VWF-repressive element in VWF-expressing and -nonexpressing cells

To conclusively demonstrate that E4BP4 mediates its repressive effect on transcription by interacting in vivo with the VWF E4BP4 element, a ChIP assay was carried out using chromatin isolated from HUVECs, and differentiated and undifferentiated Dami and HepG2 cells. E4BP4 antibodies were used to immunoprecipitate native chromatin from the different cell types, and the presence of
The role of GATA elements as positive modulators of transcriptional activity in both endothelial cells and megakaryocytes has been described, and the GATA sites at positions

Discussion

The underlying mechanisms that contribute to cell type-specific expression of the VWF gene are complex and not well understood. In our initial analysis, we compared the cell type-specific transcriptional responsiveness of the minimal VWF promoter (–91 to +229) in a variety of cell types, relative to the promoterless construct pGL2 Basic. The minimal VWF promoter is very ineffective in mediating expression in nonendothelial cells, while in contrast, a 40-fold increase in expression is observed in endothelial cells. Dami cells have been used as a megakaryocyte model and can be induced to differentiate with an associated increase in VWF production. In undifferentiated megakaryocytes, the VWF proximal promoter activity is about 10-fold greater than in non–VWF-expressing cells, and when these cells are induced to differentiate, promoter activity more than doubles. The complexities of the mechanisms responsible for increased VWF production associated with the induction of Damí cell differentiation are unresolved, and although VWF promoter activity increases, the increase in VWF production may also, in part, result from the concomitant polyploidization that occurs with differentiation.
utative GATA element at +53 have been characterized. Predictably, in primary endothelial cells, and megakaryocytes, reduction in transcriptional activity is observed in response to removal of the GATA element at nt −80. In HepG2 cells, however, a substantial increase in promoter activity was consistently observed when this GATA element was removed. It is noteworthy that the nucleotide sequences in this region are similar to those in the region of the GATA element at nt +220, and in nonepithelial cells, this sequence interacts with the NFκB transcription factor, which represses transcription by recruiting histone deacetylases. It is possible that modifications such as methylation or deacetylation at the GATA element at nt −80 repress transcription, and when these sequences are removed transcription is enhanced in the nonepithelial cells.

The presence of a repressive element at +96/+105 is indicated by the substantial increase in promoter activity that is observed when these sequences are mutated or removed from the VWF promoter construct, and this is most pronounced in cells that actively express VWF. In HepG2 cells, E4BP4 directly interacts with this element, which appears to repress basal promoter activity (Figure 7A). This inhibition may be accomplished by preventing the formation of a transcription-competent complex. E4BP4 binds DNA only when it is phosphorylated, so presumably in HepG2 cells the E4BP4 is phosphorylated and this accounts for its larger size on the Western blot. Repression by the VWF E4BP4 element is strongest in endothelial cells, and yet there is no clear evidence that E4BP4 interacts directly with this element in any of the VWF-expressing cells, even though there is an abundance of E4BP4 in the nucleus of these cells. In BAECs, the molecular mass of the protein is smaller than that seen in HepG2 cells, and it is possible that in these cells E4BP4 is not phosphorylated, and may therefore not be able to bind directly to the VWF-E4BP4 element to repress active transcription. In Dami cells, the molecular mass of the nuclear E4BP4 is substantially smaller, but analysis of the E4BP4 RNA provides no evidence that any of the different forms of E4BP4 originate from alternative splicing and all have the DNA binding motif. The VWF E4BP4 element is clearly involved in repressing VWF promoter activity since deletion or mutation of it dramatically increases VWF promoter activity. Furthermore, the ChIP assay demonstrates that in both VWF-expressing and -nonexpressing cells, E4BP4 does interact with the negative response element in vivo. E4BP4 is known to interact with several different nuclear proteins, only one of which, NC2 (Dr1), has been identified. In addition, the function of the conserved C-terminal region of E4BP4 is unknown. E4BP4 may therefore be part of a coordinated binding complex that must first interact with a DNA-associated nuclear protein before it can interact with the VWF E4BP4 binding element (Figure 7B). The presence of different sizes of E4BP4 in the nucleus of the different cell types, and the lack of direct DNA binding of E4BP4 to the VWF E4BP4 element only in cells that actively transcribe VWF, indicates that E4BP4 uses different mechanisms to repress active and basal transcription. It is interesting to note that in BAECs, HUVECs, and Dami cells there are differences in the amounts and sizes of the various forms of E4BP4, and this may contribute to differences in the level of VWF expression between different cell types. Clearly, further studies will be required to elucidate the mechanisms of E4BP4 repression of active transcription.

E4BP4 is known to interact with the TATA binding protein (TBP) NC2. NC2 binds to the TBP to repress transcription, but no complex consisting of all 3 proteins has been identified. It has been proposed that when E4BP4 does not bind DNA it sequesters NC2, thereby inhibiting its repressive effect on transcription, and that this would effectively enhance transcription (Figure 7C). In BAECs and Dami cells, there is no direct binding of E4BP4 to the VWF E4BP4 binding element, but in BAECs when this element is mutated, not only is there a restoration, but a further enhancement of VWF promoter activity. The enhanced promoter activity may result because more E4BP4 is now able to sequester additional negative regulators of transcription, and this further supports the hypothesis that a non-DNA-binding form of E4BP4 sequesters a repressor of transcription, which thereby ultimately enhances promoter activity. In HepG2 cells, where E4BP4 directly interacts with the DNA-binding element, a similar recovery of expression is not seen with the mutated E4BP4 negative response element.

E4BP4 is an inducible transcription factor and appears to play a role in diverse functions, including regulation of circadian rhythm inhibiting apoptosis in pro-B lymphocytes (reviewed in Cowell). E4BP4 may play a role in the anti-inflammatory response mediated, in part, by glucocorticoids, as dexamethasone induces the expression of E4BP4, and furthermore, genes such as Cox-2 and iNOS, which are also inducible by dexamethasone, have E4BP4 binding sites in their promoters. The data we present here further support a role for E4BP4 in regulating proinflammatory gene expression, in this instance mediating a reduction in VWF expression following its initial, positive acute-phase response. This possibility suggests that E4BP4 may play an important role in restricting increased VWF expression to short-term and transient responses.

In conclusion, this report documents, for the first time, the presence of a cis-acting repressor element between nts +96/+105
in the first, noncoding exon of the VWF gene. This element interacts with the bZIP transcriptional repressor, E4BP4, and restrains transcription of VWF in a cell type–specific manner. This repressor element is the fourth negative regulatory sequence to be characterized within the VWF promoter region. Overall, this information suggests that VWF transcription requires tight control, and that this regulatory region has evolved to significantly restrict the likelihood of constitutive protein overexpression.

References

Cell type–specific regulation of von Willebrand factor expression by the E4BP4 transcriptional repressor

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